PRODUCTION OF BIOPLASTIC FROM AGRICULTURAL WASTE

NUR NAZIRAH BINTI ZULKAFLI

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ABSTRACT

Plastic has been a vital part of our life. However, disposal of these non-degradable petroleum-derived plastic has threaten our ecosystem. Hence, extensive research has been conducted to find the best substitute to solve this problem. Much interest has been gained in developing biodegradable plastic. Among other potential biodegradable plastic, polyhydroxybutyrate (PHB) has gained much attention and developed for industrial scale production. PHB are accumulated during fermentation process and act as energy source in microbial cells. However, the major problem in commercializing PHB is its high production cost due to its expensive carbon source and tedious procedures of using pure cultures. Thus, utilization of other cheap and renewable culture has been explored. In this study, agricultural waste has been chosen as the potential carbon source for fermentation using *Bacillus subtilis* to produce PHB. The high glucose content in the sugarcane and pineapple waste juice has making it as the potential substrates. A laboratory study was conducted to screen the effect of five potential factors; temperature, pH, agitation speed, substrate to nutrient ratio and types of waste, towards the production. A total of 16 experiments have been conducted in 48 hours of cultivation time using aerobic condition in shake flask. This study had shown that temperature and agitation speed had given the most significant effect toward PHB synthesis. Temperature is known to give a significant on fermentation since different bacteria requires different temperature for optimum production. Agitation speed should be controlled since too much speed could affect the shear force hence break the bacterial cell. Interaction between factors also has been analysed and interaction between factor of temperature and agitation speed and interaction between temperature and types of waste has shown the highest contribution towards production PHB.

ABSTRAK

Plastik telah menjadi sebahagian penting dalam kehidupan seharian kita. Walau bagaimanapun, yg dihasilkan daripada petroleum ini tidak dapat dilupuskan dan telah menjejaskan ekosistem kita. Oleh itu, kajian yang menyeluruh telah dijalankan untuk mencari pengganti yang terbaik bagi menyelesaikan masalah ini dan kini, penghasilan bioplastik telah mula mendapat perhatian. Di antara bioplastik yang lain, polyhydroxybutyrate (PHB) telah mendapat perhatian dan dibangunkan untuk pengeluaran pada skala industri. PHB dikumpulkan semasa proses penapaian dan bertindak sebagai sumber tenaga dalam sel-sel mikrob. Walau bagaimanapun, masalah utama dalam mengkomersialkan PHB adalah kos pengeluaran yang tinggi disebabkan oleh sumber karbon yang mahal dan prosedur yang ketat dalam penggunaan bakteria. Oleh itu, penggunaan sumber yang murah dan boleh diperbaharui telah diterokai sehingga kini. Dalam kajian ini, sisa pertanian telah dipilih sebagai sumber karbon yang berpotensi untuk penapaian menggunakan *Bacillus subtilis* untuk menghasilkan PHB . Kandungan glukosa yang tinggi di dalam sisa tebu dan nanas telah menjadikannya sebagai substrat yang berpotensi. Kajian makmal telah dijalankan untuk melihat kesan lima faktor yang berpotensi; suhu, pH, kelajuan kacauan, nisbah kandungan substrat kepada nutrien dan jenis buah-buahan terhadap jumlah pengeluaran. Sebanyak 16 eksperimen telah dijalankan dengan tempoh 48 jam penapaian dengan bantuan oksigen di dalam kelalang kon. Kajian ini telah menunjukkan bahawa suhu dan kelajuan kacauan telah memberi kesan yang ketara terhadap penghasilan PHB. Telah diketahui umum bahawa ia memberikan kesan yang ketara terhadap penapaian kerana bakteria yang berbeza memerlukan suhu yang berbeza untuk pengeluaran yang optimum. Kelajuan kacauan perlu dikawal kerana kelajuan yang tinggi boleh menjejaskan daya ricih dan mampu memecahkan sel bakteria. Interaksi antara faktor juga telah dianalisis dan interaksi antara faktor suhu dan kelajuan kacauan menyumbangkan kesan yang paling tinggi terhadap penghasilan PHB.

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LIST OF ABBREVIATIONS

AcC	Acetyl cellulose
HPLC	High Performance Liquid Chromatography
IPP	Isotactic Polypropylene
NY11	Nylon 11
PBS	Poly(butylene succinate)
PCL	Polycaprolactone
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PHB	Polyhydroxybutyrate
PLA	Polylactide
PP	Polypropylene

1 INTRODUCTION

1.1 Overview

This chapter discuss on the outline of research conducted. There are five main parts covered in this chapter, which are the background of study, problem statement, research objective, scopes of research and the significance of study.

1.2 Background of Study and Motivation

History of plastic begins starting back in 1862 by Alexander Parkers where he has invented a moldable material made from cellulose called Parkesine. History is then followed by the invention of celluloid as a substitute for ivory in billiard ball in 1868 by John Wesley Hyatt. This was the beginning of plastic revolution but still, it is not yet applied for modern industrial use. It is then started after the production of Bakelite by American chemist, L.H. Baekeland in 1909. Made by polymerization of phenol and formaldehyde, it is a type of plastic called thermoplastic. The new uses of plastics are continually being discovered to multiple its application in regular basis. After World War II, optic wear such as optical lenses, artificial eyes and dentures of acrylic plastics have been developed. Since then, plastic has become very significant towards people due to its durability, strengths, moldability and multipurpose characteristics making people depend on it in daily life basis.

Plastics used today are originally made of petroleum based which is harmful to the environment. It takes about 100 years to completely decompose a single plastic waste. This is because they are having characteristics of high molecular weight with tightly bonded molecules. These are making them non-degradable and difficult to be disposed thus proportionally leads towards the negative impacts to the environment; includes marine life and mankind (Nisha et al., 2009).

Due to awareness on the large influence of plastic used towards the green of nature, studies have been actively conducted to look investigate the possibility of replacing the non-degradable plastic towards one that can be decomposed in a shorter time and eco-friendly as well. In addition, the use of petroleum as main source of production could be decreased and consequently able to be saved for a little bit longer even though it is depleting now. According to Nisha et al. (2009), through those researches, the concept of biodegradable plastic came as a solution for this problem. These bioplastic wastes will be decomposed by microbial degradation in the environment at proper condition such as sunlight, moisture and oxygen. More or less, through these alternatives, our environment and at the same time natural source can be preserved. Thus, this research aims to overcome the abundant plastic waste leftover by developing an eco-friendly and decomposable plastic from agricultural waste.

Even though bioplastic invention has been discovered since centuries ago, it still facing problem for an industrial production scale. One of the major limiting factors to manufacture this finding is it's highly cost of substrate which is used as carbon supply for bacteria in the fermentation process. After extensive researches conducted, a new way has been discovered to lower the cost of production in which by utilizing waste as the raw material instead of pure sugar.

Therefore, this research aims to investigate the production of PHB utilized from two different agricultural wastes which are from sugarcane and pineapple. Both peels and pulp of these two leftover were used as the carbon source in the fermentation process producing the biopolymer. Five different parameters will be screened using Two-Level Factorial Analysis by Design Expert. The factors include temperature, agitation speed, pH, types of waste and substrate to nutrient ratio.

1.3 Problem Statement

The extensive use of plastic based petroleum for over than a century has resulted a major cause towards the environment. The limited future availability of petroleum, with environment and waste managements has thus brought people's concern into more sustainable alternatives to replace petroleum-derived plastic. Synthesizing biopolymer using microbial fermentation is usually expensive with the usage of microbes, nutrient medium and substrates for carbon source. Hence, a new alternative of utilizing agro-waste to replace the original substrate has been discovered. Considering Malaysia with variety agriculture production such as pineapple, sugarcane and oil palm, wastes from these crops could be recycled to synthesize PHB. Besides that, producing a biodegradable plastic is prior to control the abundant of plastics leftover that damaging the ecosystem and nature.

1.4 Research Objective

This research is conducted to screen the production of biopolymer (PHB) in fermentation using agricultural waste by manipulating five different parameters. The measurable objectives are to determine:

1. The effect of temperature, pH, agitation speed, substrate to nutrient ratio and types of waste that enhanced the production of PHB.
2. Interaction between the parameters that contribute to the production of PHB.

1.5 Research Scope

In this research, fermentation was conducted in 500 mL conical flask. Two-Level Factorial Analysis was utilized using Design Expert version 6 software to obtain an experimental design covers the five identified parameters; temperature, pH agitation speed, substrate to nutrient ratio and types of waste. The fermentation by *Bacillus subtilis* is using sugarcane and pineapple wastes as carbon sources. PHB concentration was assessed to determine the effect of parameters studied.

1.6 Significant of Research

Microbial production of bioplastic is one of the methods to control and minimize conventional plastic usage. The only limiting factor towards an industrial scale production is its high cost of manufacturing. This research helps in promoting a way in synthesizing biopolymer by exploiting waste obtained from agricultural activities. It could assist in lowering the production cost that restrains a wide synthesis of bioplastic.

2 LITERATURE REVIEW

2.1 Overview

This chapter reviews on the experimental studies on the production of PHB via microbial fermentation. The scopes cover on the development of bioplastic, the selection of PHB for an extensive biopolymer study, potential of agricultural waste as carbon source in PHB production and the potential producer of PHB synthesis. In this chapter, the topics are reviewed to provide a basic insight to the early exposure of studies on production of PHB bioplastic from agricultural waste.

2.2 Bioplastic

Bioplastic is a form of plastic made from renewable biomass, instead of the conventional plastic that derived from petroleum. It can be divided into two groups which are biodegradable plastic or bio-based plastic (Tokiwa et al., 2009). Biodegradable plastic is made up of fossil materials while bio-based plastic is synthesized from biomass or renewable resources. It is mentioned in this study that, biodegradable plastics offer a lot of benefits and these include low accumulation of bulky plastic materials in the environment, increased soil fertility and of course reduced the cost of waste management. According to Reddy et al. (2003), there are three major types of biodegradable plastic that are currently identified; photodegradable, semi-biodegradable and completely biodegradable**.**

Photodegradable plastic has light sensitive groups that connected directly into the backbone of the polymer and act as additive. Exposure towards ultraviolet radiation from several weeks to months cause disintegration of the polymeric structure hence open it for further bacterial degradation (Kalia et al., 2000). Unfortunately, landfills lack of sunlight and thus the plastic remains non-degrade. As for semi-biodegradable plastic, it is a starch-linked plastics that connecting short fragments of polyethylene. It is still remains non-degraded since bacteria do not decompose the plastic due to the fragment of polyethylene that hindered them from attacking the starch (Johnstone, 1990). The last type of plastic is the completely degradable plastic is somehow compromising since it is produced by bacteria to form biopolymer. Some of the existing bioplastic include polyhydroxyalkanoate (PHA), polylactides (PLA), aliphatic polyesters and polysaccharides (Reddy et al., 2003).

In a research done by Tokiwa et al. (2009) on biodegradability of plastics, it is mentioned that there are inter-relationship between biodegradable plastic and bio-based plastic as shown in Figure 2.1 below.

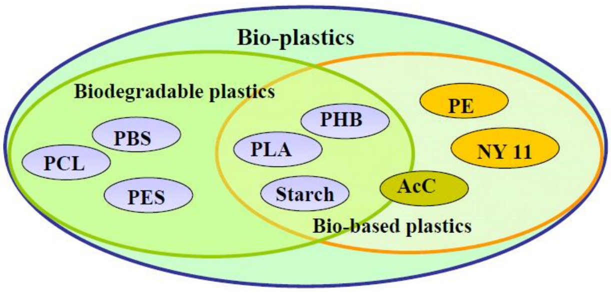

Figure 2-1: Types of Bioplastic belongs into Different Group

Based on the figure shown, even polycaprolactone (PCL), and poly (butylene succinate) (PBS) are petroleum based, however they can be degraded by microorganisms. On the other hand, polyhydroxybutyrate (PHB), polylactide (PLA) and starch blends are produced from biomass or renewable sources, hence making them biodegradable. As for polyethylene (PE) and Nylon 11 (NY11), even though they can be produced from biomass or renewable sources, they are unfortunately non-biodegradable. Acetyl cellulose (AcC) that falls in the bio-based group, it can be either biodegradable or non-biodegradable, depending on the degree of acetylation. If the AcC is having low acetylation, it can be degraded compared to those with high substitution ratios.

In order for a polymer to be decomposed, there are several factors that need to be considered, in which it includes the properties of plastic itself. Both physical and chemical properties of plastics have influenced the mechanism of biodegradation (Tokiwa, 2009). Some other components that play its important roles are surface condition (surface are, hydrophilic and hydrophobic properties), the first order and high order structures which involves chemical structure with molecular weight and glass transition temperature with melting temperature respectively.

Biodegradability of plastic has been giving much attention lately despite of other types of plastic. Limitation of landfills area for plastic disposal urges people to find various ways to overcome the problem. Hence, biodegradable plastic are seen by many as a promising solution to this problem due to their environmental-friendly characteristic. They can be derived from renewable feed stocks, thereby reducing greenhouse gas emissions.

2.3 Polyhydroxybutyrate (PHB)

Polyhydroxybutyrate (PHB) is a biodegradable polymer belongs to the polyhydroxyalkanoate (PHA) family of polyesters. It is a linear polyester of D (-)-3-hydroxybutyric acid, observed as granules in bacterial cells, mainly, Gram positive and Gram negative organisms, under microscope since 1883 by Beijerinck. According to Bregg (2006), PHB can be synthesis via three different biotechnological processes and these are fermentative production employing microorganisms, production in transgenic plants and in vitro synthesis employing isolated enzymes. However, it is most likely produced from microbial fermentation using variety of bacterial producers such as recombinant *E. coli*, *Bacillus* spp. and *Cupriavidus necator*. It is the most widely studied and also known as best characterized derivative (Sathiyanarayanan et al., 2013).

PHB is a partially crystalline polymer that has been chosen to be one of the suitable candidates to replace the conventional plastic. This is due to its mechanical properties that are comparable to those of propylene (PP). Apart from that, its versatile properties with the biodegradability characteristics have making it as an eco-friendly substitute for the synthetic polymer.

According to Wang et al (2012), PHB is a water insoluble biopolymer and relatively resistance towards hydrolytic degradation. This differentiates it from other currently available bioplastics, which are either water soluble or moisture sensitive. Table 2.1 below shows the comparison in physical properties of PHB with those of PP.

Table 2-1: Comparison of Physical Properties of PHB and PP

Properties	***PHB***	***PP***
Melting temperature, °C	175	176
Density, kg/m^3	1.250	0.905
Tensile strength, MPa	40	38
Solvent resistance	Bad	Good
UV resistance	Good	Bad

Besides that, according to Wang et al. (2012), they did mention on the other potential replacement of the existing synthetic polymer. These include polylactides (PLA), aliphatic polyesters, polysaccharides, polypropylene and other copolymers. Among the others, PHB has been given much attention than the rest due to its properties as mention above. PHB is well known for its eco-friendly properties and its complete decomposition to water and carbon dioxide by aerobic microorganism (Wang et al., 2012).

According to Purwadi (n.d.), the composition of PHA was first discovered by Lemoigne who identified the excretion of 3-hydroxybutyric acid by *Bacillus megaterium*. The role of PHB was then proposed by Macrae and Wilkinson in 1958 where they observed that *Bacillus megatarium* stored biopolymer when glucose-to-nitrogen ratio of medium was height. Hence, from that founding, they have concluded that PHB was a carbon and energy-reserve material of the bacteria itself. Figure below shows on the general structural formula of PHB:

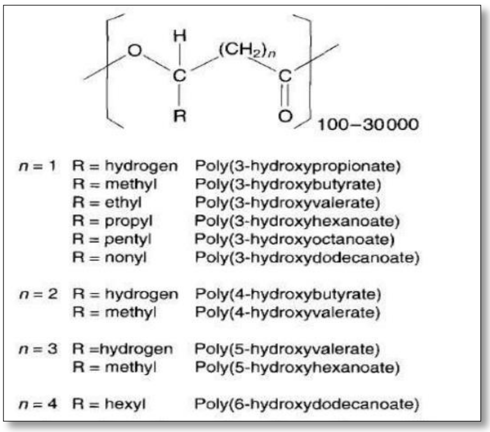

Figure 2-2: General Molecular Structure of PHB

PHB is a type of bioplastic that is having physical and mechanical properties comparable to isotactic polypropylene or known as iPP. IPP is one type of polypropylene, a thermoplastic polymer and it is different from the others in the arrangement of methyl groups that are on the same side of the chain as compared to atactic. The arrangement of methyl groups in atactic are placed randomly on both side of chain and branched. PHB is perfectly isotactic and does not having any branch and hence it flows easily during the process. Even though PHB is not water soluble, but it is 100% biodegradable in the environment when proper condition such as sunlight, moisture, and oxygen those are available.

According to Nisha et al. (2009), the degradation rate of PHB ranges from few months for anaerobic sewage up to several years in seawater besides than having low permeability for O_2, H_2O and CO_2. Therefore, PHB bioplastic have the potential to be commercially produced replacing the current plastic. This is due to its properties that are environmental friendly and the main point is that, it is capable to degrade within much less period as compared to petroleum-based plastic.

Production of PHB and other bioplastics however are known to be very expensive since they are involving expensive carbon source. Apart from that, both the upstream processing and downstream processing were also contribute to the high production cost Therefore, studies have been actively conducted to find the suitable alternative to replace the original glucose. This is where they have come out with the utilization of waste included agricultural waste, sugar industrial wastewater and cafeteria waste.

2.4 *Waste as Carbon Source*

It is formerly known that the major restriction in commercializing bioplastic is their high production cost. According to Reddy et al. (2003), the cost of PHA using natural producer *A. eutrophus* is US$16 per kg, which is 18 times more expensive than the conventional plastic. This is enough to hamper the commercial application and wide use of biopolymer. In fermentation practise, it is a must for bacteria to get an adequate sugar sources as the main carbon supply for them to produce product. In this case, to get the fine raw sugar for industrial scale PHB production is very costly, in addition with operational cost itself, it is somehow difficult to produce biopolymer industrially. Lee (1996) reported that the price of the product ultimately depends on the cost of substrate, PHA yield on the substrate and efficiency of product formulation in downstream processing.

Extensive researches have been done to discover new finding where the use of available cheap residues can be considered. Malaysia has been reported to be the fifth largest country in Asia that producing agricultural waste annually and most of them were dump without being utilized. Some of the available waste that has the potential for substrates replacement includes sugarcane bagasse, oil palm front juice, fruit peels and pulp. Hence, it is a benefit to exploit this advantage to at least minimize the production cost of PHB.

In a research done by Shivakumar (2012), he has studied on several agro-industrial residues as carbon substrate for PHB production. Agricultural wastes involved are soya flour, bagasse, molasses, rice bran and ragi bran using *Bacillus thuringiensis*. He found that these wastes capable to synthesis PHB biopolymer in different quantities. Soya flour capable to produce PHB up to 0.89 g/L, while using molasses is 0.47 g/L. As for ragi bran is 0.21 g/L PHB produced with bagasse and wheat bran are the least with 0.09 g/L and 0.07 g/L PHB produced respectively.

According to Fukui & Doi (1998), plant oils such as olive, corn and palm oils were good substrates for PHB production using *A. eutrophus*. Olive oil shows a significant effect towards the production of PHB where it produce up to 1g over 50 mL of fermentation medium. Apart from that, oleic acid was also good to be used as carbon source with *Pseudomonas putida* as the producer and has been used instead of alkanes since it exhibits less toxicity (Lee et al., 2000). In a research done by Preethi et al. (2012) using Jambul seed as the substrate for PHB production, it is found that the maximum level of PHA accumulation was observed using *Ralstonia eutropha* with 41.7% of PHA produced.

In order to obtain an optimum product secretion, the raw substrate used need to undergo pre-treatment first. Van-Thuoc et al. (2007) reported in order to employ agro-industrial residues as fermentation substrates; it should be subjected to hydrolysis step first in order to release the easily metabolized sugars. Pandey et al. (2009) also have reported the same in his study using *Bacillus sphaericus* NCIM 5149 where it shows an increment in synthesis of PHB since pre-treatment helps to utilize those wastes into simpler sugar, directly for bioconversion into PHB.

2.5 Microorganism

Microbial fermentation of PHB production requires producer for product secretion. There are two major groups of bacteria that have the potential to utilize raw substrates for the production of PHB. These bacteria grow based on the culture conditions required for PHB synthesis. As for the first group, microbes belong to this group requires the limitation of essential nutrients such as nitrogen, magnesium, sulphur and phosphorus for the synthesizing of PHB from an excess carbon sources (Purwadi, n.d.). Bacteria that fall in this group include *R. eutropha*, *Protomonas oleovorans* and *Protomonas extorquens*. The second group of bacteria belongs to those that do not require limitation of nutrient for PHB synthesis. For these particular types of bacteria, biopolymer is accumulated during the growth phase of bacteria and microbes included *Alcaligenes latus* and recombinant *E. coli*. It is nearly 300 bacteria have been identified to have the ability of producing PHB bioplastic and below are three selected bacteria that proved to have the ability to synthesize PHB.

2.5.1 Recombinant *E. coli*

Recombinant *E. coli* bacteria are one of the few bacteria that has used for industrial production of PHB. Apart from that, among the others, it has been chosen to be the best and better commercial producer of PHB since it can use a wider range of cheap carbon sources. PHB polymer produced also is much easier to be extracted and purified from these particular bacteria than the others. According to Nikel et al. (2006), PHB is efficiently produced by a recombinant strain that grown aerobically in a fed batch cultures, using medium supplied with agro-waste. Based on the research done by Nikel et al. (2006), cells have accumulated PHB to 72.9% of their cell dry weight, reaching a productivity of 2.13g PHB per litre per hour. Physical analysis on the recovered PHB shows that its molecular weight is similar to the PHB produced by *Azotobacter* spp. and higher than bioplastic produced from *Cupriavidus necator***.**

Apart from that, in another studies made by Zhang et al. (1994), on the production of polyhydroxyalkanoates (PHA) in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains, they found that the usage of recombinant *E.coli* as the producer capable to obtain intracellular polymer accumulations to a level as high as 95% biopolymer per cell dry weight with glucose, lactose or whey as the substrate. Together with the finding, they also discover that *E. coli* genetics capable to develop strains which synthesize the copolymer PHB-co-V that can be lysed by only the method of osmotic shock. It contains plasmid that does not need to be initially stabilised by antibiotics in the medium.

Several advantages have been identified for PHB production using recombinant *E. coli* bacteria, and these include its capability to produce PHB bioplastic within only 24 hours. This is compared to the other non-engineered producers, it take up to three days for the production to occur. Apart from that, a wide range of substrates also have been identified to produce PHB using recombinant *E. coli* and these include whey, agricultural wastes and molasses. This was discussed in Chee et al. (2013), where a recombinant *E. coli* strains has been reported to produce PHB on molasses as carbon source. The final dry cell weight, product content and productivity were determined to be 39.5%, 80% and 1 g/L/h respectively.

2.5.2 Cupriavidus necator

Cupriavidus necator also formerly known as *Ralstonia eutropha*, is one of the commonly known bacteria that accumulates PHA in a nutrient-limited condition excluded carbon source. It is a stable organism that formerly known to accumulate PHB with high productivity. Apart from recombinant *E. coli*, *Cupriavidus necator* is also commonly used and studied extensively to accumulate a large amount of PHB, around 80% (w/w) of cell dry weight from simple carbon source (Purwadi, n.d.).

The capability of *Cupriavidus necator* to produce a large amount of bioplastic has been proved by Verlinden (2011). In his study on production of PHA from waste frying oil, he found that the bacteria produced PHB from waste frying oil and the concentration, 1.2 g/L, obtained using the substrate was as high as the concentration that can be obtained from glucose. This has shown its capability on utilizing waste and still producing a significant amount of PHB. Higher PHB yield has been achieved in Fiorese et al. (2009) study's in which the yield is about 95% with 84% of purity when extracted from *Cupriavidus necator* cells at 130°C from 30 minutes without involving any pre-treatment. In another study made by Taniguchi et al. (2003), he has reported that waste plant oils and waste tallow has been discovered to successfully produced PHB with high yield by *Cupriavidus necator*. Currently, among other potential producers, PHB bacterial fermentation using *Cupriavidus necator* is the most cost-effective fermentation process, high productivity and is used widely in industrial processes.

2.5.3 Bacillus subtilis

Bacillus subtilis, is started to be paid attention as the potential producer of PHB after its performance in production of metabolites, bioremediation and generation of bioenergy. It is formerly recognised in the industrial scale production of amino acids, recombinant proteins and fine chemicals but never been tried for the biopolymers production (Singh et al., 2009).

Bacillus subtilis also known as grass bacilli are Gram-positive bacteria and well known bacteria species that are capable to grow within many environments (Earl, Losick & Kolter, 2008). Their capabilities that can be isolated from many environments, making them seems like they are broadly adapted to grow in various environmental condition. *Bacillus subtilis*, like other members of *bacillus* species, may form a highly resistant dormant endospores when undergo nutrient deprivation and other environmental stresses. It has been reported that among potential *Bacillus* spp., the PHB yield vary from 11% to 69% w/w of dry cell weight. Singh et al. (2009) has reported that *Bacillus subtilis* capable to synthesis PHB biopolymer but it is not suitable for an industrial production scale.

3 MATERIAL AND METHODS

3.1 Overview

This chapter explains in detail the research procedures of production of PHB bioplastic from sugarcane and pineapple peels and pulps. This research consists of five main parts which are factor identification, experimental designation, sample preparation, incubation and analysis. Waste juices obtained from both sugarcane and pineapple wastes were incubated with *Bacillus subtilis* bacteria in a flask. The samples were incubated according to the data that have been manipulated from the five factors affecting PHB production. As for the sample analysis, HPLC equipment has been used to determine the PHB concentration by comparing the peak area of samples with PHB standard's.

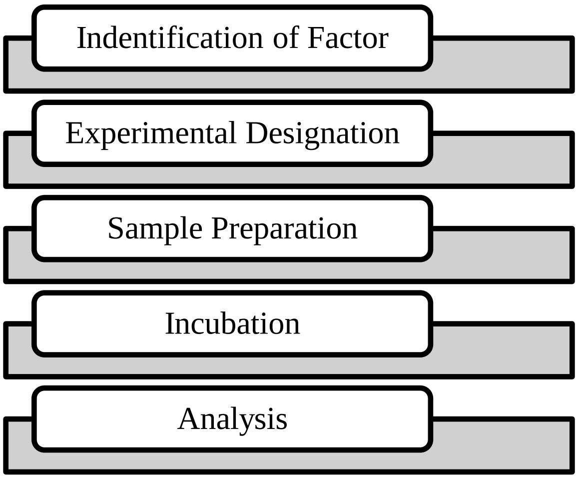

Figure 3-1: Work Flow of Research

3.2 Identification of Factor

There are five factors that have been identified to be studied as the parameters that could affect the production of PHB. All five factors; temperature, pH, agitation speed, types of waste and substrate to nutrient ratio, will used in the experimental designation using Two-Level Factorial Analysis utilized by Design Expert software. This software will arrange the experiments manipulating all five components so that a range of suitable parameter for the experiment could be obtained. Figure below shows on the five factors affecting the production of PHB.

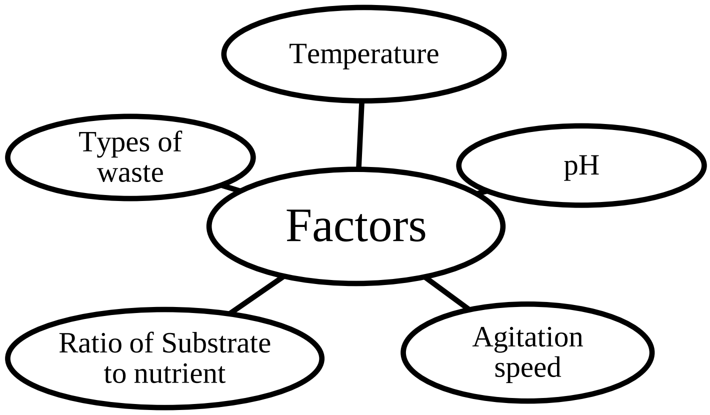

Figure 3-2: Factors Affecting the Production of PHB